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FORM PTO-1390 (REV. 9-2001)		U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE		ATTORNEY'S DOCKET NUMBER 18484-002320US	
TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371				U.S. APPLICATION NO. (If known, see 37 CFR 1.5) 10/069323	
INTERNATIONAL APPLICATION NO. PCT/US00/22801		INTERNATIONAL FILING DATE 18 August 2000		PRIORITY DATE CLAIMED 20 August 1999	
TITLE OF INVENTION METHODS FOR <i>IN VIVO</i> GENE DELIVERY TO SITES OF CARTILAGE DAMAGE					
APPLICANT(S) FOR DO/EO/US GHIVIZZANI, Steven C.; EVANS, Christopher H.; ROBBINS, Paul D.					
Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:					
1. <input checked="" type="checkbox"/> This is a FIRST submission of items concerning a filing under 35 U.S.C. 371. 2. <input type="checkbox"/> This is a SECOND or SUBSEQUENT submission of items concerning a filing under 36 U.S.C. 371. 3. <input checked="" type="checkbox"/> This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include items (5), (6), (9) and (21) indicated below. 4. <input checked="" type="checkbox"/> The US has been elected by the expiration of 19 months from the priority date (Article 31). 5. <input checked="" type="checkbox"/> A copy of the International Application as filed (35 U.S.C. 37(c)(2)) a. <input type="checkbox"/> is attached hereto (required only if not communicated by the International Bureau). b. <input type="checkbox"/> has been communicated by the International Bureau c. <input checked="" type="checkbox"/> is not required, as the application was filed in the United States Receiving Office (RO/US). 6. <input type="checkbox"/> An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)). a. <input type="checkbox"/> is attached hereto. b. <input type="checkbox"/> has been previously submitted under 35 U.S.C. 154(d)(4). 7. <input checked="" type="checkbox"/> Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3)). a. <input type="checkbox"/> are attached hereto (required only if not communicated by the International Bureau). b. <input type="checkbox"/> have been communicated by the International Bureau. c. <input type="checkbox"/> have not been made; however, the time limit for making such amendments has NOT expired. d. <input checked="" type="checkbox"/> have not been made and will not be made. 8. <input type="checkbox"/> An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)). 9. <input type="checkbox"/> An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)). 10. <input type="checkbox"/> An English language translation of the annexes of the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)). Items 11 to 20 below concern document(s) or information included: 11. <input type="checkbox"/> An Information Disclosure Statement under 37 CFR 1.97 and 1.98. 12. <input type="checkbox"/> An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included. 13. <input type="checkbox"/> A FIRST preliminary amendment. 14. <input type="checkbox"/> A SECOND or SUBSEQUENT preliminary amendment. 15. <input type="checkbox"/> A substitute specification. 16. <input type="checkbox"/> A change of power of attorney and/or address letter. 17. <input type="checkbox"/> A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821 - 1.825. 18. <input type="checkbox"/> A second copy of the published international application under 36 U.S.C. 19. <input type="checkbox"/> A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4). 20. <input checked="" type="checkbox"/> Other items or information:					
Correspondence Transmitted to the United States Patent and Trademark Office with Express Mail Label EL 951826316 US					

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U.S. Application no. (if known, see 37 CFR 1.55) 10/069323		INTERNATIONAL APPLICATION NO PCT/US00/22801		ATTORNEY'S DOCKET NUMBER 18484-002320US	
21. <input checked="" type="checkbox"/> The following fees are submitted:				CALCULATIONS PTO USE ONLY	
BASIC NATIONAL FEE (37 CFR 1.492(A) (1) - (5)):					
Neither international preliminary examination fee (37 CFR 1.492) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO				\$1040.00	
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search report prepared by the EPO of JPO				\$890.00	
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO				\$740.00	
International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4)				\$710.00	
International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)(4)				\$100.00	
ENTER APPROPRIATE BASIC FEE AMOUNT =				\$100.00	
Surcharge of \$130.00 for furnishing the oath or declaration later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(e)).				\$	
CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE	\$	
Total claims	32 - 20 =	12	x \$18.00	\$216.00	
Independent claims	5 - 3 =	2	x \$84.00	\$168.00	
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ 280.00	\$0.00	
TOTAL OF ABOVE CALCULATIONS =				\$484.00	
<input checked="" type="checkbox"/> Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above are reduced by 1/2.				+	
SUBTOTAL =				\$242.00	
Processing fee of \$130.00 for furnishing the English translation later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(f)).				\$	
TOTAL NATIONAL FEE =					
Fee for recording the enclosed assignment (37 CFR 1.2(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). \$40.00 per property +				\$	
TOTAL FEES ENCLOSED =				\$242.00	
				Amount to be refunded:	
				\$	
				charged:	
				\$	
a. <input type="checkbox"/> A check in the amount of \$_____ to cover the above fees is enclosed. b. <input checked="" type="checkbox"/> Please charge my Deposit Account No. <u>20-1430</u> in the amount of <u>\$242.00</u> to cover the above fees. c. <input checked="" type="checkbox"/> The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. <u>20-1430</u> . A duplicate copy of this sheet is enclosed. d. <input type="checkbox"/> Fees are to be charged to a credit card. WARNING: Information on this form may become public. Credit card information should not be included on this form. Provide credit card information and authorization on PTO-2038					
NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.					
SEND ALL CORRESPONDENCE TO:				February 20, 2002	
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Application Data Sheet**Application Information**

Application number::	10/069,323
Filing Date::	02/20/02
Application Type::	Regular
Subject Matter::	Utility
Suggested classification::	
Suggested Group Art Unit::	
CD-ROM or CD-R??::	
Number of CD disks::	
Number of copies of CDs::	
Sequence Submission::	
Computer Readable Form (CRF)?::	
Number of copies of CRF::	
Title::	Methods for In Vivo Gene Delivery to Sites of Cartilage Damage
Attorney Docket Number::	018484-002320US
Request for Early Publication::	No
Request for Non-Publication::	No
Suggested Drawing Figure::	
Total Drawing Sheets::	5
Small Entity?::	No
Latin name::	
Variety denomination name::	
Petition included?::	No
Petition Type::	
Licensed US Govt. Agency::	
Contract or Grant Numbers One::	
Secrecy Order in Parent Appl.::	No

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Correspondence Information

Correspondence Customer Number:: 20350

Representative Information

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Domestic Priority Information

Application::	Continuity Type::	Parent Application::	Parent Filing Date::
This Application	National Stage of	PCT/US00/22801	08/18/00

Foreign Priority Information

Country::	Application number::	Filing Date::
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METHODS FOR *IN VIVO* GENE DELIVERY TO SITES OF CARTILAGE DAMAGE

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PRIORITY

This application is a continuation-in-part of and claims priority from United States Provisional Application Serial No. 60/150,118 filed August 20, 1999.

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FIELD OF THE INVENTION

The present invention relates to improved methods for transferring genes to cells *in vivo*. In particular, it relates to the use of gene therapy techniques to transfect subchondral cells in animal joints. Such methods can be used to introduce marker genes into the cells, or to introduce genes into the cells that promote chondrogenesis in the joint.

15

BACKGROUND OF THE INVENTION

It has long been recognized that damaged cartilage lacks the ability to repair itself effectively. This is true regardless of the cause of the damage, *i.e.*, whether due to trauma or to an inflammatory or degenerative disease such as rheumatoid arthritis or osteoarthritis. One likely reason for the resistance of cartilage to repair is that adult articular cartilage lacks a blood supply, lymphatic drainage, and innervation. Hence, the cells within the cartilage that could potentially promote cartilage growth, called chondrocytes, are shut off from entities likely to stimulate healing, such as circulating factors, hormones, or cells. In addition, the chondrocytes are embedded within a dense matrix, and are thus unable to migrate or traffic, thus preventing their travel to sites of damage.

25

One type of cartilage defect, called a partial-thickness defect, refers to cartilage damage that does not extend to the subchondral bone. Partial-thickness defects are especially resistant to healing, and the chondrocytes in such cases exhibit almost no response to the defect. Full thickness defects, which extend into the subchondral bone, evoke a greater response, but nevertheless fail to produce a satisfactory repair tissue. Full thickness defects often degenerate into osteoarthritis, and in many cases ultimately require the surgical replacement of the joint. Clearly, improved methods for treating both partial thickness and full thickness defects are needed.

30

Several attempts to stimulate cartilage repair have focused on the chondrocytes, which reside in the cartilage and are responsible for maintaining the extracellular matrix of the cartilage. The extracellular matrix provides the mechanical properties of the cartilage, such as its shock absorbing, load bearing, and protective properties. Chondrocytes are capable of both producing and degrading the extracellular matrix, and are thought to, in a healthy joint, maintain a proper balance between matrix production and degradation so as to maintain normal cartilage. It is thought that some diseases, such as osteoarthritis, result from the loss of this balance in chondrocyte metabolism, resulting in an overall net loss of cartilage.

One method that has shown some efficacy in treating cartilage injury is the administration of cultured chondrocyte cells to joints with damaged cartilage. For example, Brittberg *et al.*, (*New Eng J Med* 331:889 (1994)) introduced cultured, autologous chondrocytes into defects of the articular cartilage. While this treatment showed some positive results, the success was variable and limited, and the long-term results are not yet known. Further, the drawbacks of this method include the need to harvest autologous cartilage and the need for a second, open surgical procedure to implant the cells.

Some efforts have been made to influence the behavior and/or growth of chondrocytes *in vivo* by administering purified growth factors, cytokines or cytokine inhibitors/antagonists. Because systemic delivery of proteins to chondrocytes depends upon vascular perfusion of the synovium, which occurs generally by passive diffusion and which is particularly inefficient for large molecules such as proteins, attempts have been made to inject proteins directly into the joint. Despite some encouraging results, short half-lives limit the use of these factors, necessitating repeat application, risk of toxicity if administered at high doses, and the need for an appropriate delivery system that provides the factors in their bioactive forms.

To overcome such problems, gene transfer methods have been suggested for the local treatment of joint disorders. However, the obvious problem remains that the transfer of genes to articular cartilage chondrocytes is inhibited by the presence of a very abundant, dense extracellular matrix. Although this would be predicted to rule out gene delivery *in vivo* except in cases of advanced disease, where the matrix is depleted, Tomita *et al.*, (*Arthritis Rheum* 40:901 (1997)) have demonstrated liposome and Sendai viral

vector mediated marker gene transfer to the articular chondrocytes of normal rat knee joints following intra-articular injection.

In certain cases, chondrocytes have been cultured and transfected *in vitro*, and the transfected cells transplanted into a joint with a cartilage defect (*see, e.g., Kang et al.,*
5 (*1997) Osteoarthritis and Cartilage* 5:139-143; Glorioso *et al.*, U.S. Patent Application Serial No. 08/466,932, filed April 6, 1995; Baragi *et al.*, (*1997) Osteoarthritis and Cartilage* 5:275-282; Doherty *et al.*, (*1998) Osteoarthritis and Cartilage*, 6:153-160; Baragi *et al.*, (*1995) J.Clin. Invest.* 96:1995).

Additional cell types that have been implicated in chondrogenesis include the
10 chondroprogenitor cells, including the mesenchymal stem cells. Such cells are located within the bone marrow (Wakitani, S. *et al.*, *J. Bone Jt Surg* 76A:579 (1994)), periosteum (Oleksyszyn, J. *et al.*, *Inflamm Res* 45:464 (1996)) and, perhaps, elsewhere (Hunziker, E.B. *et al.*, *J Bone Jt Surg* 78A:721 (1996); Iwata, H. *et al.*, *Clin Orthop Rel Res* 296:295 (1993)). Mesenchymal stem cells can differentiate into any of a number of cell types,
15 including cartilage, bone, and tendon. In general, the inaccessibility of chondroprogenitor and mesenchymal stem cells has limited therapeutic methods involving such cells to *ex vivo* approaches (*see, e.g., Glorioso et al.*, WO9639196; Allay, J.A. *et al.*, *Human Gene Ther* 8:1417 (1997); Balk, M.L. *et al.*, *Bone* 21:7 (1997)). For example, these cell types have been isolated from bone marrow, grown in culture, and introduced into damaged
20 joints as a potential strategy for joint repair. In addition, some attempts have been made to recruit these cells for cartilage repair by drilling, abrading, or producing "microfractures" into the subchondral bone (Mitchell and Shepard (1976) *J. Bone Joint Surg.* 58-A:230; Rae and Noble (1989) *J. Bone Joint Surg.* 71-B:534; Kim *et al.*, (1991) *J. Bone Joint Surg.* 73-A:1301). These attempts have failed to produce extensive
25 cartilage repair, however.

Despite these advances, new formulations and methods for improving the efficiency of gene transfer to cells *in vivo*, particularly for local gene delivery methods, is desirable. The present invention addresses these and other needs.

SUMMARY OF THE INVENTION

The present invention provides methods of expressing heterologous proteins in subchondral cells *in vivo*. In general, the present methods involve exposing subchondral

cells *in vivo* and transfecting the cells with a polynucleotide encoding the heterologous protein, whereby the polynucleotide is expressed.

In numerous embodiments, the present methods involve providing a heterologous protein in a subchondral cell in a joint of a mammal by creating a perforation in a subchondral bone of the joint, and introducing a polynucleotide encoding the heterologous protein into the perforation, whereby a subchondral cell, *e.g.*, a chondroprogenitor or mesenchymal stem cell that is exposed by or localized to the perforation internalizes the polynucleotide and expresses the heterologous protein.

The perforation can be produced by a number of means, *e.g.*, drilling, microfracture, or abrasion. Preferably, the perforation is produced by one of these means arthroscopically.

In certain embodiments, the polynucleotide is introduced into the perforation in the subchondral bone using an artificial matrix, for example collagen.

In preferred embodiments, the heterologous protein is a chondrogenesis-promoting polypeptide. In particularly preferred embodiments, the chondrogenesis-promoting polypeptide is a growth factor (*e.g.*, TGF- β), a cartilage-derived morphogenetic factor, a cytokine inhibitor (*e.g.*, an interleukin-1 receptor antagonist), or a proteinase inhibitor.

The polynucleotide can be introduced using a variety of means, for example using an adenoviral vector, a retroviral vector, formulated plasmid DNA, naked DNA, an adeno-associated viral vector, a herpes simplex viral vector, or other non-viral DNA formulations.

In certain embodiments, the mammal has arthritis, osteoarthritis, osteochondritis dissecans, avascular necrosis, or has undergone an injury to the joint.

Pharmaceutical formulations and kits for practicing the present methods are also provided.

DEFINITIONS

Unless defined otherwise herein, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Singleton, *et al.*, DICTIONARY OF MICROBIOLOGY AND MOLECULAR BIOLOGY, 2D ED., John Wiley and Sons, New York (1994), and Hale & Marham, THE HARPER COLLINS DICTIONARY OF BIOLOGY, Harper Perennial, NY (1991)

provide one of skill with a general dictionary of many of the terms used in this invention. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are described. Numeric ranges are inclusive of the numbers defining the range.

5 Unless otherwise indicated, nucleic acids are written left to right in 5' to 3' orientation; amino acid sequences are written left to right in amino to carboxyl orientation, respectively. The headings provided herein are not limitations of the various aspects or embodiments of the invention which can be had by reference to the specification as a whole. Accordingly, the terms defined immediately below are more fully defined by
10 reference to the specification as a whole.

The phrase "expression cassette" refers to a nucleic acid sequence to be introduced into a cell and contains the nucleic acid sequence to be transcribed and a promoter to direct the transcription. The promoter can either be homologous, *i.e.*, occurring naturally to direct the expression of the desired nucleic acid or heterologous, *i.e.*, occurring
15 naturally to direct the expression of a nucleic acid derived from a gene other than the desired nucleic acid. Fusion genes with heterologous promoter sequences are desirable, *e.g.*, for regulating expression of encoded proteins. In some instances, the promoter may constitutively bind transcription factors and RNA Polymerase II. In other instances, a heterologous promoter may be desirable because it has sequences that bind transcription
20 factors the naturally occurring promoter lacks. In addition to a promoter, an expression cassette preferably contains termination sequences and a poly A+ signal for, *e.g.*, mRNA stabilization.

The term "identical" in the context of two polypeptide sequences refers to the residues in the two sequences that are the same when aligned for maximum
25 correspondence, as measured using one of the following "sequence comparison algorithms." The phrase "substantially identical" in the context of two polypeptides refers to the residues in the two sequences that have at least 60% identity when aligned for maximum correspondence over a domain of the protein. Optimal alignment of sequences for comparison can be conducted, *e.g.*, by the local homology algorithm of
30 Smith & Waterman, *Adv. Appl. Math.* 2:482 (1981), by the homology alignment algorithm of Needleman & Wunsch, *J. Mol. Biol.* 48:443 (1970), by the search for similarity method of Pearson & Lipman, *Proc. Nat'l. Acad. Sci. USA* 85:2444 (1988), by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and

TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, WI), or by inspection.

One example of a useful algorithm is PILEUP. PILEUP creates a multiple sequence alignment from a group of related sequences using progressive, pairwise
5 alignments. It can also plot a tree showing the clustering relationships used to create the alignment. PILEUP uses a simplification of the progressive alignment method of Feng & Doolittle, *J. Mol. Evol.* 35:351-360 (1987). The method used is similar to the method described by Higgins & Sharp, *CABIOS* 5:151-153 (1989). The program can align up to 300 sequences of a maximum length of 5,000. The multiple alignment procedure begins
10 with the pairwise alignment of the two most similar sequences, producing a cluster of two aligned sequences. This cluster can then be aligned to the next most related sequence or cluster of aligned sequences. Two clusters of sequences can be aligned by a simple extension of the pairwise alignment of two individual sequences. The final alignment is achieved by a series of progressive, pairwise alignments. The program can also be used
15 to plot a dendrogram or tree representation of clustering relationships. The program is run by designating specific sequences and their amino acid coordinates for regions of sequence comparison.

Another example of algorithm that is suitable for determining sequence similarity is the BLAST algorithm, which is described in Altschul, *et al.*, *J. Mol. Biol.* 215:403-410
20 (1990). Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/>). This algorithm involves first identifying high scoring sequence pairs (HSPs) by identifying short words of length W in the query sequence that either match or satisfy some positive-valued threshold score T when aligned with a word of the same length in a database
25 sequence. T is referred to as the neighborhood word score threshold (Altschul, *et al.*, *supra*). These initial neighborhood word hits act as seeds for initiating searches to find longer HSPs containing them. The word hits are extended in both directions along each sequence for as far as the cumulative alignment score can be increased. Extension of the word hits in each direction are halted when: the cumulative alignment score falls off by
30 the quantity X from its maximum achieved value; the cumulative score goes to zero or below, due to the accumulation of one or more negative-scoring residue alignments; or the end of either sequence is reached. The BLAST algorithm parameters W, T, and X determine the sensitivity and speed of the alignment. The BLAST program uses as

defaults a wordlength (W) of 11, the BLOSUM62 scoring matrix (see Henikoff & Henikoff, *Proc. Natl. Acad. Sci. USA* 89:10915 (1989)) alignments (B) of 50, expectation (E) of 10, M⁺5, N⁻4, and a comparison of both strands.

The BLAST algorithm performs a statistical analysis of the similarity between two sequences (see, e.g., Karlin & Altschul, *Proc. Nat'l. Acad. Sci. USA* 90:5873-5787 (1993)). One measure of similarity provided by the BLAST algorithm is the smallest sum probability (P(N)), which provides an indication of the probability by which a match between two nucleotide or amino acid sequences would occur by chance. For example, a nucleic acid is considered similar to a ribonuclease nucleic acid if the smallest sum probability in a comparison of the test nucleic acid to a ribonuclease nucleic acid is less than about 0.1, more preferably less than about 0.01, and most preferably less than about 0.001. Where the test nucleic acid encodes a ribonuclease polypeptide, it is considered similar to a specified ribonuclease nucleic acid if the comparison results in a smallest sum probability of less than about 0.5, and more preferably less than about 0.2.

Another indication that two polypeptides are substantially identical is that the first polypeptide is immunologically cross-reactive with the second polypeptide. Thus, a polypeptide is substantially identical to a second polypeptide, for example, where the two peptides differ only by a conservative substitution.

The terms "isolated," "purified" or "biologically pure" refer to material that is substantially or essentially free from components which normally accompany it as found in its native state. Purity and homogeneity are typically determined using analytical chemistry techniques such as polyacrylamide gel electrophoresis or high performance liquid chromatography. A polypeptide that is the predominant species present in a preparation is substantially purified. The term "purified" denotes that a polypeptide gives rise to essentially one band in an electrophoretic gel. Particularly, it means that the polypeptide is at least 85% pure, more preferably at least 95% pure, and most preferably at least 99% pure.

As used herein, the terms "nucleic acid," "nucleic acid sequence," or "polynucleotide" refer to any deoxyribonucleotide or ribonucleotide sequence in, e.g., single-stranded, double-stranded or triplex form. The terms encompass nucleic acids, e.g., oligonucleotides, containing known naturally occurring nucleotides, analogues of natural nucleotides, and mixtures thereof. The term also encompasses nucleic-acid-like structures with synthetic backbones. DNA backbone analogues provided by the invention

include phosphodiester, phosphorothioate, phosphorodithioate, methyl-phosphonate, amino phosphonate, phosphor-amidate, alkyl phosphotriester, sulfamate, 3'-thioacetal, methylene (methylimino), 3'-N-carbamate, morpholino carbamate, and peptide nucleic acids (PNAs); see *Oligonucleotides and Analogues, a Practical Approach*, edited by F. Eckstein, IRL Press at Oxford University Press (1991); *Antisense Strategies*, Annals of the New York Academy of Sciences, Volume 600, Eds. Baserga and Denhardt (NYAS 1992); Milligan (1993) *J. Med. Chem.* 36:1923-1937; *Antisense Research and Applications* (1993, CRC Press). PNAs contain non-ionic backbones, such as N-(2-aminoethyl) glycine units. Phosphorothioate linkages are described in WO 97/03211; WO 96/39154; Mata (1997) *Toxicol. Appl. Pharmacol.* 144:189-197. Other synthetic backbones encompassed by the term include methyl-phosphonate linkages or alternating methylphosphonate and phosphodiester linkages (Strauss-Soukup (1997) *Biochemistry* 36:8692-8698), and benzylphosphonate linkages (Samstag (1996) *Antisense Nucleic Acid Drug Dev.* 6:153-156). The "nucleic acids," or "polynucleotides" of the present invention can comprise any of a number of nucleotide analogs, *i.e.*, nucleotides modified to include fluorescent moieties, radioactive isotopes, biotin, digoxigenin, 2,4-Dinitrophenyl (DNP), 5' Bromo-2'-deoxyUridine (BrdU), chemically reactive side chains, alternative bases such as inosine, uridine, or others. "Nucleic acids," or "polynucleotides," can be of any length, including short polymers such as oligonucleotides, as well as longer sequences such as amplification fragments, restriction fragments, plasmids, and other vectors. The "nucleic acids" or "polynucleotides" can be made through any standard method, such as by chemical or other *in vitro* synthesis, by amplification reactions, or by isolation from cells, and can include naturally-occurring as well as recombinant sequences.

As used herein, the term "pharmaceutically acceptable carrier" includes any suitable pharmaceutical excipient, including, *e.g.*, water, saline, phosphate buffered saline, Hank's solution, Ringer's solution, dextrose/saline, glucose, lactose, or sucrose solutions, starch, cellulose, talc, gelatin, malt, rice, flour, chalk, silica gel, magnesium stearate, sodium stearate, glycerol monostearate, dried skim milk, glycerol, propylene glycol, ethanol, and the like.

The term "recombinant" when used with reference to a protein indicates that a cell expresses a peptide or protein encoded by a nucleic acid whose origin is exogenous to the cell. Recombinant cells can express genes that are not found within the native (non-recombinant) form of the cell. Recombinant cells can also express genes found in the

native form of the cell wherein the genes are re-introduced into the cell by artificial means, for example under the control of a heterologous promoter or using a plasmid or viral vector.

The phrase "reduces the symptoms of" or "ameliorating" or "ameliorate" refers to any indicia of success in the treatment of a pathology or condition, including any objective or subjective parameter such as abatement, remission or diminishing of symptoms or an improvement in a patient's physical or mental well-being. Amelioration of symptoms can be based on objective or subjective parameters; including the results of a physical examination and/or a psychiatric evaluation.

The phrase "specifically (or selectively) binds" refers to a binding reaction that is determinative of the presence of a polypeptide in a heterogeneous population of polypeptides and other compounds. Thus, under designated binding conditions, the specified polypeptides bind to a particular compound at least two times the background and do not substantially bind in a significant amount to other compounds present in the sample.

"Transfection," or "transduction," as used herein means the delivery of exogenous nucleic acid molecules to a cell, either *in vivo* or *in vitro*, whereby the nucleic acid is taken up by the cell and is functional within the cell. A cell that has taken up the exogenous nucleic acid is referred to as a "host cell," "target cell," "transduced cell," or "transfected cell." A nucleic acid is functional within a host cell when it is capable of functioning as intended. Usually, the exogenous nucleic acid will comprise an expression cassette which includes DNA coding for a gene of interest, with appropriate regulatory elements, which will have the intended function if the DNA is transcribed and translated, thereby causing the host cell to produce the peptide or protein encoded therein. DNA may encode a protein lacking in the transfected cell, or produced in insufficient quantity or less active form, or secreted, where it may have an effect on cells other than the transfected cell. Other examples of exogenous nucleic acid to be delivered include, *e.g.*, antisense oligonucleotides, mRNA ribozymes, or DNA encoding antisense RNA or ribozymes. Nucleic acids of interest also include DNA coding for a cellular factor which, when expressed, activates the expression of an endogenous gene. A cell can be "transfected" or "transduced" using any of the methods provided herein, including those using viral based vectors, naked DNA, or DNA formulations such as liposomal formulations.

A “polypeptide,” or “protein” is a polymer comprising monomers that are interconnected by peptide linkages. The monomers are most often the twenty naturally occurring amino acids (*see, e.g.* Alberts *et al*, (1989) Molecular Biology of the Cell, Garland Publishing, Inc.), but may also include amino acid analogs or other compounds that can be linked using peptide chemistry. A “heterologous protein” refers to any polypeptide that has been introduced into a cell or that is expressed from a polynucleotide that has been introduced into a cell. A “heterologous protein” can comprise a different sequence than a naturally expressed protein, can have the same sequence but be expressed at a different level or with altered timing or regulation than an endogenous protein, or can represent a polynucleotide identical to an endogenous gene that has been introduced, *e.g.*, to replace a defective endogenous gene or to increase the natural level of expression of a protein.

The term “cartilage” refers to connective tissue comprising collagen and proteoglycan, particularly chondroitin sulphate. Cartilage is typically produced by chondrocytes that come to lie in small lacunae surrounded by the matrix they have secreted.

The term “intra-articular” refers to the interior of a joint, *e.g.*, knee, elbow, shoulder, ankle, wrist, *etc.* Thus, an intra-articular injection is an injection into the space between the bones of a joint. In the knee, “intra-articular” refers to the space between the femur and the tibia, behind and surrounding the patella.

An "artificial matrix," or "implanted matrix" refers to any substance in which molecules, cells, or other materials can be embedded. Commonly, an "artificial" or "implanted" "matrix" will comprise a regular framework within which such items can be embedded, and will be composed of a biological or other polymer, *e.g.*, collagen.

25 “Subchondral bone” refers to any bone located beneath cartilage.

“Subchondral cell” refers to any cell, such as a chondroprogenitor or mesenchymal stem cell, that is exposed by or localized to a perforation introduced or existing in a subchondral bone. The cell can be directly exposed to the exterior of the animal, or can simply be placed in fluidic or other contact with the exterior, thereby allowing the application of material, *e.g.*, a polynucleotide formulation, to the cell.

A “chondroprogenitor cell” is any cell that is capable of promoting chondrogenesis, *e.g.*, by differentiating into a chondrocyte, by promoting cartilage synthesis, by inhibiting cartilage degradation, and the like.

“Mesenchymal stem cells” refer to cells, present within the bone marrow, that can differentiate to form cells associated with any of a number of tissue types, such as cartilage, bone, fat, muscle, and other tissues.

The term “arthritis” refers to any of a number of inflammatory or degenerative conditions that affects joints. “Arthritis” can result from any of a number of causes, including infection, autoimmune disorders, or injury.

The term “osteoarthritis” refers to a noninflammatory degenerative joint disease characterized by degeneration of the articular cartilage, hypertrophy of bone at the margins and changes in the synovial membrane. Also known as degenerative joint disease, osteoarthritis is the most common form of arthritis.

“Cartilage injury” refers to any damage or lesion in a cartilage tissue. In cases of articular cartilage, injuries can occur through any of a number of means, including from direct trauma resulting, *e.g.*, in damage from rotational forces, shearing injuries, secondary effects of surgery, and the like, or from any of a number of degenerative or inflammatory injuries such as osteoarthritis. A “cartilage injury” can occur in any part of the cartilage, such as medial, lateral, *etc.*, and can refer to any type of lesion or damage, such as fibrillation, discoloration, softness, cracks, tears, craters, *etc.* In addition, a “cartilage injury” can be “full-thickness,” meaning that the injury extends through the cartilage and into the subchondral bone, or can be “partial thickness,” meaning that the injury does not extend completely through the cartilage into the subchondral bone.

A “perforation” in a bone refers to any hole, breach, fracture, aperture, piercing, or any other opening that places cells within the bone, blood stream, or bone marrow, *e.g.* chondroprogenitor cells, into contiguous contact with the exterior of the bone. For example, a “perforation” in a subchondral bone will expose the chondroprogenitor cells within the marrow of the bone, thereby allowing the application of molecules, *e.g.*, nucleic acids, to such cells. A “perforation” can be introduced into a bone by any of a number of mechanisms, including, but not limited to, drilling, abrasion, microfracturing, and piercing. It will be appreciated that a perforation can be created expressly for the practice of the present invention, or can be pre-existing, *e.g.*, the result of a previous injury or degenerative disease or disorder.

A “chondrogenesis-promoting polypeptide,” or a “chondrogenic polypeptide,” is any polypeptide that causes a net increase in cartilage synthesis, chondrocyte levels, or any property typical of healthy cartilage or articulations, *e.g.*, the resilience, shock

absorbing ability, *etc.* of the cartilage. Such factors can include growth factors that stimulate growth and/or proliferation of chondroprogenitor cells or chondrocytes, factors that promote the differentiation of chondroprogenitor cells into chondrocytes, *e.g.*, cartilage-derived morphogenetic factors, cytokine inhibitors, *e.g.*, interleukin-1 receptor antagonists, *etc.*

"Localizing" a polynucleotide to a joint, *e.g.*, by introducing the polynucleotide into the joint using a mixture of collagen and the polynucleotide, refers to the introduction of the polynucleotide into the joint in such a way that the local concentration of the polynucleotide remains at a significant level for an extended period of time, *i.e.*, the polynucleotide does not immediately diffuse away from the site of administration. "Localizing" a polynucleotide to a joint facilitates the transfection of cells within the joint after the initial moment of administration, *e.g.*, cells migrating into the joint subsequent to administration of the polynucleotide can be readily transfected.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows luciferase expression following implantation of adenoviral vectors into cartilage defects in joints.

Figure 2 shows luciferase expression following non-viral delivery of the luciferase gene into cartilage defects in joints.

Figure 3 shows production of insulin-like growth factor 1 (IGF-1) by transduced chondrocytes and its effects on matrix synthesis. Figure 3A shows IGF-1 concentrations in conditioned media following transduction with the IGF-1 gene or addition of IGF-1 protein. Figure 3B shows the effects of IGF-1 protein and gene on the synthesis of proteoglycans by chondrocytes. Figure 3C shows the effects of IGF-1 protein and gene on the synthesis of collagen and noncollagenous proteins by chondrocytes.

Figure 4 shows the additive effects of growth factor (insulin-like growth factor 1 [IGF-1] and transforming growth factor β 1 [TGF β 1]) gene combinations on proteoglycan synthesis by chondrocytes.

Figure 5 shows the effects of growth factor (transforming growth factor $\beta 1$ [TGF $\beta 1$] and insulin-like growth factor 1 [IGF-1]) genes on proteoglycan synthesis in the presence of interleukin-1 (IL-1).

5 DETAILED DESCRIPTION OF THE INVENTION AND PREFERRED
 EMBODIMENTS

The present invention provides methods of transfecting subchondral cells *in vivo*. Using these methods, subchondral cells within a bone or bone marrow, or cells within the bloodstream, are exposed by introducing a perforation into a subchondral bone in a joint.
10 These exposed subchondral cells are then transfected with a polynucleotide encoding a protein of interest, leading to expression of the polynucleotide *in vivo*. Such methods are useful for numerous applications, including providing a marker for subchondral cells *in vivo*, and promoting chondrogenesis by expressing a chondrogenesis-promoting polynucleotide, *e.g.*, a growth factor, in the cells.

15 In general, the methods entail multiple steps, including providing a perforation into a subchondral bone by any method, *e.g.*, drilling, piercing, abrasion, or microfracturing the bone, thereby exposing the cells. Such methods are preferably performed arthroscopically. Once a perforation is obtained, the exposed cells are preferably contacted with the polynucleotide by formulating the polynucleotide in an
20 artificial matrix, *e.g.* a collagen matrix, and introducing the matrix-polynucleotide formulation into the perforation. The nucleic acid will typically encode a marker gene or a gene that promotes the growth-, proliferation-, and/or chondrogenic differentiation of subchondral cells, or a gene that promotes the *in vivo* synthesis of cartilage components, *e.g.*, collagen, thereby promoting cartilage growth in a joint.

25 The present methods can be used to mark subchondral cells in a joint, thereby providing a method for monitoring the growth, proliferation, survival, localization, *etc.*, of these cells *in vivo*. Such methods are useful in any animal, including humans and animal models, *e.g.*, to monitor the efficacy of a treatment for a cartilage defect, such as a method aimed to promote the growth of cells, including chondroprogenitor or
30 mesenchymal stem cells, *in vivo*. The ability to monitor subchondral cells *in vivo* is also useful to monitor the progression of a disease or condition, *e.g.*, osteoarthritis, over time. This ability provides important information regarding the nature and severity of a disease that can be used to inform decisions regarding optimal treatment for the disease.

The present methods can also be used to introduce chondrogenesis-promoting nucleotides into subchondral cells in a joint, *e.g.*, a joint with a cartilage injury, of an animal such as a human or an animal model. For example, a gene that promotes chondrogenic differentiation of subchondral cells, or of the growth and/or proliferation of subchondral cells can be used. In addition, genes that promote the production of cartilage components, *e.g.*, collagen, or that inhibit the degradation of cartilage components, can be used. Such methods are useful to promote cartilage growth in joints with cartilage defects or injury, and are thus useful as methods of treatment for such conditions.

Any of the herein-described methods can be performed on any animal, preferably on mammals including, but not limited to, humans.

In vivo gene delivery

As noted above, the methods of the invention involve delivery of genes to cells exposed by a perforation in a subchondral bone in a joint. The methods also typically involve the use of techniques to introduce at least one perforation in the subchondral bone to expose cells present in the bone to the vectors of the invention. To practice the invention, cells can be exposed by any method that penetrates the subchondral bone, allowing bleeding and/or entry of bone marrow into the lesion. Methods with which to do this include drilling, abrasion, and microfracture, *e.g.*, using an arthroscopic awl to make one or more perforations in the subchondral bone. All of these methods are preferably performed arthroscopically.

Several approaches for introducing nucleic acids into the exposed cells *in vivo* are known. Most methods involve delivery using viral, *e.g.*, adenoviral, or plasmid vectors.

A convenient method of introducing the polynucleotides into subchondral cells involves the use of viral vectors, *e.g.*, adenoviral vector mediated gene delivery (*see, e.g.* Chen *et al.* (1994) *Proc. Nat'l. Acad. Sci. USA* 91: 3054-3057; Tong *et al.* (1996) *Gynecol. Oncol.* 61: 175-179; Clayman *et al.* (1995) *Cancer Res.* 55: 1-6; O'Malley *et al.* (1995) *Cancer Res.* 55: 1080-1085; Hwang *et al.* (1995) *Am. J. Respir. Cell Mol. Biol.* 13: 7-16; Haddada *et al.* (1995) *Curr. Top. Microbiol. Immunol.* 199 (Pt. 3): 297-306; Addison *et al.* (1995) *Proc. Nat'l. Acad. Sci. USA* 92: 8522-8526; Colak *et al.* (1995) *Brain Res.* 691: 76-82; Crystal (1995) *Science* 270: 404-410; Elshami *et al.* (1996) *Human Gene Ther.* 7: 141-148; Vincent *et al.* (1996) *J. Neurosurg.* 85: 648-654); and retroviral vectors (*see, e.g.*, Marx *et al.* *Hum Gene Ther* 1999 May 1;10(7):1163-73).

Mason *et al.*, *Gene Ther* 1998 Aug;5(8):1098-104). In addition, replication-defective retroviral vectors harboring a therapeutic polynucleotide sequence as part of the retroviral genome have also been used, particularly with regard to simple MuLV vectors. *See, e.g.*, Miller *et al.* (1990) *Mol. Cell. Biol.* 10:4239 (1990); Kolberg (1992) *J. NIH Res.* 4:43, and Cornetta *et al.* *Hum. Gene Ther.* 2:215 (1991)). Other suitable retroviral vectors include lentiviruses (Klimatcheva *et al.*, (1999) *Front Biosci* 4:D481-96). Other viral vectors that can be used in the present invention include vectors derived from adeno-associated viruses (Bueler (1999) *Biol Chem* 380(6):613-22; Robbins and Ghivizzani (1998) *Pharmacol Ther* 80(1):35-47), herpes simplex viruses (Krisky *et al.*, (1998) *Gene Ther* 5(11):1517-30), and others.

Plasmid vectors are typically delivered as "naked" DNA or combined with various transfection-facilitating agents. Numerous studies have demonstrated the direct administration of naked DNA, *e.g.*, plasmid DNA, to cells *in vivo* (*see, e.g.*, Wolff, *Neuromuscul Disord* 1997 Jul;7(5):314-8, Nomura *et al.*, *Gene Ther.* 1999 Jan;6(1):121-9). For certain applications it is possible to coat the DNA onto small particles and project genes into cells using a device known as a gene gun.

Plasmid DNA can also be combined with any of a number of transfection-facilitating agents. When combined with any such agents, the plasmid DNA is referred to herein as "formulated plasmid DNA." The most commonly used transfection facilitating agents for plasmid DNA *in vivo* has been charged and/or neutral lipids (Debs and Zhu (1993) WO 93/24640 and U.S. Pat. No. 5,641,662; Debs U.S. Pat. No. 5,756,353; Debs and Zhu Published EP Appl. No. 93903386; Mannino and Gould-Fogerite (1988) *BioTechniques* 6(7): 682-691; Rose U.S. Pat No. 5,279,833; Brigham (1991) WO 91/06309 and U.S. Pat. 5,676,954; and Felgner *et al.* (1987) *Proc. Natl. Acad. Sci. USA* 84: 7413-7414). Additional useful liposome-mediated DNA transfer methods, other than the references noted above, are described in US Patent Nos. 5,049,386, US 4,946,787; and US 4,897,355; PCT publications WO 91/17424, WO 91/16024; Wang and Huang, 1987, *Biochem. Biophys. Res. Commun.* 147: 980; Wang and Huang, 1989, *Biochemistry* 28: 9508; Litzinger and Huang, 1992, *Biochem. Biophys. Acta* 1113:201; Gao and Huang, 1991, *Biochem. Biophys. Res. Commun.* 179: 280. Immunoliposomes have been described as carriers of exogenous polynucleotides (Wang and Huang, 1987, *Proc. Natl. Acad. Sci. U.S.A.* 84:7851; Trubetskoy *et al.*, 1992, *Biochem. Biophys. Acta* 1131:311) and may have improved cell type specificity as compared to liposomes by virtue of the

inclusion of specific antibodies which presumably bind to surface antigens on specific cell types. Behr *et al.*, 1989, *Proc. Natl Acad. Sci. U.S.A.* 86:6982 report using lipopolyamine as a reagent to mediate transfection itself, without the necessity of any additional phospholipid to form liposomes.

5 Lipid carriers usually contain a cationic lipid and a neutral lipid. Most *in vivo* transfection protocols involve forming liposomes made up of a mixture of cationic and neutral lipid and complexing the mixture with a nucleic acid. The neutral lipid is often helpful in maintaining a stable lipid bilayer in liposomes used to make the nucleic acid:lipid complexes, and can significantly affect transfection efficiency. Liposomes may
10 have a single lipid bilayer (unilamellar) or more than one bilayer (multilamellar). They are generally categorized according to size, where those having diameters up to about 50 to 80 nm are termed "small" and those greater than about 80 to 1000 nm, or larger, are termed "large." Thus, liposomes are typically referred to as large unilamellar vesicles (LUVs), multilamellar vesicles (MLVs) or small unilamellar vesicles (SUVs).

15 Cationic liposomes are typically mixed with polyanionic compounds (including nucleic acids) for delivery to cells. Complexes form by charge interactions between the cationic lipid components and the negative charges of the polyanionic compounds.

A wide variety of liposomal formulations are known and commercially available and can be tested in the assays of the present invention for precipitation, DNA protection,
20 pH effects and the like. Because liposomal formulations are widely available, no attempt will be made here to describe the synthesis of liposomes in general. Two references which describe a number of therapeutic formulations and methods are WO 96/40962 and WO 96/40963.

The lipid carriers of the invention will generally be a mixture of cationic lipid and
25 neutral helper lipid in a molar ratio of from about 3:1 to 1:3, preferably about 1:1. The lipid carriers may include cholesterol, DOPE (dioleoylphosphatidylethanolamine), DLPE (1,2-dilauroyl-sn-glycero-3-phosphoethanolamine), DOTMA (N-(2,3-dioleoyloxy)propyl-N,N,N-triethylammonium chloride), or DiPPE (Diphytenoyl phosphatidyl ethanolamine) alone or in combination as the helper lipid, or may include additional non-cationic helper
30 lipids, which may be either anionic or neutral lipids. Usually, the lipid carriers will have, as the lipid components, a single cationic lipid and a single neutral lipid, preferably in approximately equimolar amounts. Lipid mixtures typically are prepared in chloroform, dried, and rehydrated in, *e.g.*, 5% dextrose in water or a physiologic buffer to form

liposomes. Low ionic strength solutions are preferred. Liposomes may be LUVs, MLVs, or SUVs. Usually, the liposomes formed upon rehydration are predominantly MLVs, and SUVs are formed from them by sonication or by extrusion through membranes with pore sizes ranging from 50 to 600nm to reduce their size. Most preferably, the liposomes are extruded through a series of membranes with decreasing pore sizes, *e.g.*, 400nm, 200nm and 50nm.

The resulting liposomes are mixed with a nucleic acid solution with constant agitation to form the cationic lipid-nucleic acid transfection complexes.

Cationic lipid-nucleic acid transfection complexes can be prepared in various formulations depending on the target cells to be transfected. While a range of lipid-nucleic acid complex formulations will be effective in cell transfection, optimal conditions are determined empirically in the desired system. Lipid carrier compositions are evaluated, *e.g.*, by their ability to deliver a reporter gene (*e.g.*, CAT, which encodes chloramphenicol acetyltransferase, luciferase, β -galactosidase, or GFP) *in vitro*, or *in vivo* to a given tissue type in an animal, or in assays which test stability, protection of nucleic acids, and the like.

The lipid mixtures are complexed with nucleic acids in different ratios depending on the target cell type, generally ranging from about 6:1 to 1:20 μg nucleic acid:nmole cationic lipid.

Non-lipid material (such as biological molecules being delivered to an animal cell) can be conjugated to the lipid carriers through a linking group to one or more hydrophobic groups, *e.g.*, using alkyl chains containing from about 12 to 20 carbon atoms, either prior or subsequent to vesicle formation. Various linking groups can be used for joining the lipid chains to the compound. Functionalities of particular interest include thioethers, disulfides, carboxamides, alkylamines, ethers, and the like, used individually or in combination. The particular manner of linking the compound to a lipid group is not a critical part of this invention, and the literature provides a great variety of such methods.

Formulations suitable for administration include aqueous isotonic sterile injection solutions, which can contain antioxidants, buffers, bacteriostats, and solutes that render the formulation isotonic with the blood of the intended recipient, and aqueous and non-
aqueous sterile suspensions that can include suspending agents, solubilizers, thickening agents, stabilizers, and preservatives.

For administration, the formulations of the present invention can be administered at a rate determined by the LD-50 (or other measure of toxicity) of the cell type, and the side-effects at various concentrations, as applied to the mass and overall health of the mammal. Administration can be accomplished via single or divided doses. The vectors of this invention can supplement other treatments for a condition by known conventional therapy, including medical or surgical approaches.

In preferred embodiments, the vectors of the invention are delivered to the site of exposed subchondral cells in an artificial matrix or plug to facilitate transfection efficiency. Means for preparing and delivering the artificial matrix are described below.

Genes

Any of a variety of genes or gene products can be used in the present invention. For example, any marker gene, growth factor, cytokine antagonist or proteinase inhibitor can be used. In general, any polypeptide that marks cells *in vivo*, or that stimulates chondrocyte or chondroprogenitor cell growth, proliferation, chondrogenic differentiation, matrix synthesis, or any other aspect of chondrogenesis can be used. In addition, any of a variety of intracellular proteins, as well as RNA species, can be used to regulate gene expression therapeutically.

Any of a number of marker genes can also be used in the present invention, where “marker gene” refers to a gene whose expression allows the detection of expressing cells through any of a variety of means, *e.g.* visual detection. Such marker genes allow the visualization of cells for monitoring the growth, proliferation, or survival of subchondral cells *in vivo*. Such markers could be used, *e.g.*, to monitor the efficacy of a clinical treatment, or to monitor the progress of a disease over time. Suitable such marker genes include β -galactosidase, alkaline phosphatase, alcohol dehydrogenase, GFP (Ikawa *et al.*, (1999) *Curr Top Dev Biol* 44:1-20; Haseloff (1999) *Methods Cell Biol* 58:139-51; Tsien (1998) *Annu Rev Biochem* 67:509-44), firefly luciferase (Naylor (1999) *Biochem Pharmacol* 58(5):749-57), and others.

In preferred embodiments, nucleic acids encoding growth factors are used, where “growth factor” refers to any polypeptide that modulates the rate and/or extent of cellular, tissue, or organismal growth or repair. Growth factors can thus be used to facilitate the repair or regeneration of cartilage by stimulating growth and/or proliferation in subchondral cells, matrix production, and/or the chondrocytic differentiation of

chondroprogenitor or mesenchymal stem cells, either implanted or locally available in the joint. Also preferred is the introduction of genes encoding inhibitors or antagonists of cytokines and growth factors that are involved in the degeneration of cartilage. Equally useful are inhibitors of proteases that directly degrade matrix molecules or indirectly act by degrading growth factors, which would otherwise aid the repair or regeneration processes. In numerous embodiments, multiple such genes are used.

Any of a variety of growth factors can be used to increase matrix deposition *in vivo* (Flechtenmacher, J. *et al.*, *Arthritis Rheum* 39:1896 (1996); Glansbeek, H.L. *et al.*, *Arthritis Rheum* 40:1020 (1997); Morales, T.I. *et al.*, *J Biol Chem* 263:12828 (1988); Tyler, J.A. *Biochem J* 260:543 (1989)). In particularly preferred embodiment, transforming growth factor- β (TGF- β), such as TGF- β 1, TGF- β 2, or TGF- β 3, or a homolog, derivative, or fragment thereof is used (*see, e.g.*, Lafeber, FPJG *et al.*, *Br J Rheum* 32:281 (1993)). Also preferred is IGF-1, TGF-alpha, at least one fibroblast growth factor, the bone morphogenetic proteins (BMPs) 2-12 (*see, e.g.*, Schmitt *et al.*, *J Orthop Res* 1999 Mar;17(2):269-78; Cho and Blitz, *Curr Opin Genet Dev* 1998 Aug;8(4):443-9), cartilage-derived growth and differentiation factors or cartilage-derived morphogenetic protein (*see, e.g.*, Reddi, *Microsc Res Tech* 1998 Oct 15;43(2):131-6; Chang *et al.*, *J Biol Chem* 1994 Nov 11;269(45):28227-34; Tsumaki *et al.*, (1999), *J Cell Biol* 1999 Jan 11;144(1):161-73); members of the hedgehog family (*see, e.g.*, Bianco *et al.*, *Matrix Biol* 1998 Jul;17(3):185-92; Ingham, *EMBO J* 1998 Jul 1;17(13):3505-11), and others.

It has been shown that interleukin-1 activates chondrocytes and induces cartilage breakdown *in vivo*. Additionally, interleukin-1 inhibits cartilaginous matrix synthesis by chondrocytes, thereby suppressing repair of cartilage. Interleukin-1 also induces bone resorption and thus may account for the loss of bone density seen in rheumatoid arthritis. As a result, in some embodiments of this invention, polynucleotides will be used that encode proteins that inhibit one or more effects of interleukin-1 on joints, such as cartilage breakdown (*see, e.g.*, Caron, J.P. *et al.*, *Arthritis Rheum* 39:1535 (1996), and U.S. Patent No. 5,858,355). For example, the interleukin-1 receptor antagonist (IL-1Ra, or IRAP) can be used. This gene is capable of binding to and neutralizing interleukin-1 *in vivo*. Soluble forms of IL-1R can also be used.

A number of additional cytokines or cytokine antagonists can also be used to inhibit the catabolic activities of chondrocytes, including IL-13 (Jovanovic, D. *et al.*,

Osteoarthritis Cart6:40 (1997)), IL-4 (Yeh, L.A. *et al.*, *J Rheumatol* 22:1740 (1995)), and soluble forms of IL-1 and TNF receptors. Biological inhibitors of the proteinases produced by osteoarthritic chondrocytes include the tissue inhibitors of metalloproteinases (TIMPs), plasminogen activator inhibitors, serpins and α_2 -macroglobulin, among others (Cawston, T. *et al.*, *Ann NY Acad Sci* 723:75 (1994); Oleksyszyn, J. *et al.*, *Inflamm Res* 45:464 (1996); Travis, J. *et al.*, *Ann Rev Biochem* 52:655 (1983)). Also suitable for the present invention is IL-10, *e.g.*, vIL-10 (*see, e.g.*, Lechman *et al.*, (1999) *J Immunol* 163(4):2202-8), inhibitors of NO synthase, and Ik- β (*see, e.g.*, Wulczyn *et al.*, *J Mol Med* 1996 Dec;74(12):749-69).

In numerous embodiments, the present methods will be used to inhibit the expression of one or more genes. Such inhibition can be accomplished, *e.g.*, using antisense or ribozyme technology (*see, e.g.*, Couture, L.A. *et al.*, *Trends Genet* 12:510 (1996)). Antisense or ribozyme nucleic acids can be administered directly or by delivery of genes encoding antisense or ribozyme molecules.

Vectors

Preparation of various polynucleotides and vectors useful in the present invention are well known. General texts which describe methods of making recombinant nucleic acids include Sambrook *et al.*, Molecular Cloning - A Laboratory Manual (2nd Ed.), Vol. 1-3, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1989 ("Sambrook"); Current Protocols in Molecular Biology, F.M. Ausubel *et al.*, eds., Current Protocols, a joint venture between Greene Publishing Associates, Inc. and John Wiley & Sons, Inc., (supplemented through 1998) ("Ausubel"); and Berger and Kimmel, Guide to Molecular Cloning Techniques, Methods in Enzymology volume 152 Academic Press, Inc., San Diego, CA (Berger).

Product information from manufacturers of biological reagents and experimental equipment also provide information useful in known biological methods such as cloning. Such manufacturers include the SIGMA chemical company (Saint Louis, MO), R&D systems (Minneapolis, MN), Pharmacia LKB Biotechnology (Piscataway, NJ), CLONTECH Laboratories, Inc. (Palo Alto, CA), Chem Genes Corp., Aldrich Chemical Company (Milwaukee, WI), Glen Research, Inc., GIBCO BRL Life Technologies, Inc. (Gaithersburg, MD), Fluka Chemica-Biochemika Analytika (Fluka Chemie AG, Buchs, Switzerland), Invitrogen, San Diego, CA, Applied Biosystems (Foster City, CA), Digene

Diagnostics, Inc. (Beltsville, MD) as well as many other commercial sources known to one of skill. These commercial suppliers produce extensive catalogues of compounds, products, kits, techniques and the like for performing a variety of standard methods.

In numerous embodiments of this invention, nucleic acids will be inserted into
5 vectors using standard molecular biological techniques. Vectors may be used at multiple stages of the practice of the invention, including for subcloning nucleic acids encoding, *e.g.*, components of proteins or additional elements controlling protein expression, vector selectability, *etc.* Vectors may also be used to maintain or amplify the nucleic acids, for example by inserting the vector into prokaryotic or eukaryotic cells and growing the cells
10 in culture.

In some embodiments, introduction of the present nucleic acids into cells *in vivo* will be achieved using viral vectors. For example, vectors derived from retroviruses, adenovirus, adeno-associated virus, or herpes simplex virus have found use in human clinical trials, and can be used herein. Retroviruses and adeno-associated viruses offer
15 particular advantages in the pursuit of long term gene expression because they integrate their genetic material into the chromosomal DNA of the cells they infect. For use as vectors, the viruses are genetically disabled so that they cannot replicate or cause disease, yet can still transfer genes effectively. A type of retrovirus known as a lentivirus is able to transduce non-dividing cells, and can also be used.

Vectors derived from adenoviruses are increasingly popular, because the viral
20 vectors can be grown to high titer, are able to contain large segments of DNA, and are highly infectious to both dividing and non-dividing cells. The latter property facilitates their use during *in vivo* protocols where the gene is delivered directly to the patient. *See*, Rosenfeld, M. A., *et al.*, 1992, *Cell*, 68:143155; Jaffe, H. A. *et al.*, 1992, *Nature Genetics*
25 1:372-378; Lemarchand, P. *et al.*, 1992, *Proc. Natl. Acad. Sci. USA*, 89:6482-6486. Adeno-associated virus (AAV) can also be used, as it causes no known diseases and integrates at a precise location into the chromosomal DNA of the cells it infects. *See, e.g.*, Xiao *et al.*, *J Virol* 1999 May;73(5):3994-4003; Prince, *Pathology* 1998 Nov;30(4):335-47; Rabinowitz and Samulski, *Curr Opin Biotechnol* 1998 Oct;9(5):470-5).

For mammalian host cells, viral-based and nonviral, *e.g.*, plasmid-based,
30 expression systems are provided. Nonviral vectors and systems include plasmids and episomal vectors, typically with an expression cassette for expressing a protein or RNA, and human artificial chromosomes (*see, e.g.*, Harrington *et al.*, 1997, *Nat Genet* 15:345).

For example, plasmids useful for expression of polynucleotides and polypeptides in mammalian (e.g., human) cells include pcDNA3.1/His, pEBVHis A, B & C, (Invitrogen, San Diego CA), MPSV vectors, others described in the Invitrogen 1997 Catalog (Invitrogen Inc, San Diego CA), which is incorporated herein in its entirety by reference, and numerous others known in the art.

Useful viral vectors include vectors based on retroviruses, adenoviruses, adeno-associated viruses, herpes viruses, vectors based on SV40, papilloma virus, HBP Epstein Barr virus, vaccinia virus vectors and Semliki Forest virus (SFV). SFV and vaccinia vectors are discussed generally in Ausubel *et al.*, *supra*, Ch. 16. These vectors are often made up of two components, a modified viral genome and a coat structure surrounding it (see generally, Smith, 1995, *Ann. Rev. Microbiol.* 49: 807), although sometimes viral vectors are introduced in naked form or coated with proteins other than viral proteins. However, the viral nucleic acid in a vector may be changed in many ways, for example, when designed for gene therapy. The goals of these changes are to disable growth of the virus in target cells while maintaining its ability to grow in vector form in available packaging or helper cells, to provide space within the viral genome for insertion of exogenous DNA sequences, and to incorporate new sequences that encode and enable appropriate expression of the gene of interest.

Viral vector nucleic acids generally comprise two components: essential cis-acting viral sequences for replication and packaging in a helper line and the transcription unit for the exogenous gene. Other viral functions are expressed in trans in a specific packaging or helper cell line. Adenoviral vectors (e.g., for use in human gene therapy) are described in, e.g., Rosenfeld *et al.*, 1992, *Cell* 68: 143; PCT publications WO 94/12650; 94/12649; and 94/12629. In cases where an adenovirus is used as an expression vector, a sequence may be ligated into an adenovirus transcription/translation complex consisting of the late promoter and tripartite leader sequence. Insertion in a nonessential E1 or E3 region of the viral genome will result in a viable virus capable of expressing in infected host cells (Logan and Shenk, 1984, *Proc. Natl. Acad. Sci.*, 81:3655). Replication-defective retroviral vectors harboring a therapeutic polynucleotide sequence as part of the retroviral genome are described in, e.g., Miller *et al.*, 1990, *Mol. Cell. Biol.* 10: 4239; Kolberg, 1992, *J. NIH Res.* 4: 43; and Cornetta *et al.*, 1991, *Hum. Gene Ther.* 2: 215. In certain embodiments, the surface of the virus can be coated, e.g., by covalent attachment, with polyethylene glycol (PEG; see, e.g., O'Riordan *et al.*,

(1999) *Hum Gene Ther.* 10(8):1349-58.). Such "PEGylation" of viruses can impart various benefits, including increasing the infectivity of the virus, and lowering the host immune response to the virus.

While any promoter capable of driving gene expression *in vivo* or *in vitro* can be used, in mammalian cell systems promoters from mammalian genes or from mammalian viruses are often appropriate. Suitable promoters may be constitutive, cell type-specific, stage-specific, and/or modulable or regulable (*e.g.*, by hormones such as glucocorticoids). Useful promoters include, but are not limited to, the metallothionein promoter, the constitutive adenovirus major late promoter, the dexamethasone-inducible MMTV promoter, the SV40 promoter, the MRP polIII promoter, the constitutive MPSV promoter, the tetracycline - inducible CMV promoter (such as the human immediate - early CMV promoter), the constitutive CMV promoter, and promoter - enhancer combinations known in the art.

Artificial Matrices

As noted above, while the vectors of this invention can be administered in any format, the vectors are typically formulated in an artificial matrix to enhance transfection efficiency. Although the matrices used to deliver the polynucleotide formulations according to the present methods are comprised of natural components, *e.g.*, collagen, they are referred to herein as "artificial matrices" to distinguish them from endogenous matrix, *e.g.*, collagen matrix, present in natural cartilage. Preferably the artificial matrix is easy to handle, porous, spongy, malleable, and resorbable by the body. A number of suitable artificial matrix materials are known to those of skill in the art. For example, type I collagen can be used as the artificial matrix material. Other artificial matrices can be made, *e.g.*, from type II collagen or other types of collagen, hyaluronan or other glycosaminoglycans, fibrin, various synthetic biodegradable polymers, and mixtures of the above. *See, e.g.*, Riesle *et al.*, *J Cell Biochem* 1998 Dec 1;71(3):313-27.

In preferred embodiments, the vector solution is mixed with a collagen solution and allowed to gel. In other embodiments, collagen can be dipped in a solution of plasmid DNA and inserted into the perforated bone (*see, e.g.*, Fang *et al.*, *Proc Natl Acad Sci U S A* 93(12):5753-8); Bonadio and Fang, WO 98/22492).

Pharmaceutical Compositions.

5 those skilled in the art and are described in more detail in such publications as
Remington's Pharmaceutical Science, 15th ed., Mack Publishing Company, Easton,
 Pennsylvania (1980).

disease or injury and its complications. An amount adequate to accomplish this is defined as a "therapeutically effective dose." Amounts effective for this use will depend upon the severity of the disease and the general state of the patient's health. Single or multiple administrations of the compositions may be administered depending on the dosage and frequency as required and tolerated by the patient. In any event, the composition should provide a sufficient quantity of the molecules described herein to effectively treat the patient.

20 population) and the ED₅₀ (the dose therapeutically effective in 50% of the population) using procedures presented herein and those otherwise known to those of skill in the art. The therapeutic index (LD₅₀/ED₅₀) can be determined from these experiments.

typical pharmaceutical composition of the invention would depend on the particular gene, patient, medical condition, pharmaceutical composition, and other factors, but will typically be from about 10 ng to about 10 mg, preferably between about 10 µg and about 1 mg of nucleic acid, *e.g.*, plasmid or vector DNA. For administration of adenoviral formulations, between about 1×10^5 and 1×10^9 pfu is used, preferably between about 1×10^6 and 1×10^8 pfu. For treatment of any of the causes of cartilage damage described herein, the vectors of the invention can be administered either alone or in conjunction with other well-known therapies.

Cartilage damage

The present methods can be used to express any of a number of chondrogenesis-promoting proteins in subchondral cells exposed by a perforation in a subchondral bone in a joint. Such chondrogenesis-promoting proteins can be used to promote the growth and/or proliferation of the cells (*e.g.*, using growth factors), the chondrogenic differentiation of the cells (*e.g.*, using morphogenetic or other factors), matrix production by the cells (*e.g.*, using morphogenetic, growth, and/or other factors), and can be used to inhibit cartilage degradation in the joint (*e.g.*, using cytokine and/or proteinase inhibitors). As such, the present methods can be used to promote chondrogenesis in a joint, where "chondrogenesis" refers to the ability to promote cartilage growth in a joint, *e.g.*, by promoting chondrocyte growth, proliferation, and/or differentiation, by promoting matrix synthesis, or inhibiting matrix degradation in a joint. In this way, the present methods can be used to promote chondrogenesis within a damaged joint, and can thus be used to treat any of a large number of conditions.

Essentially, any condition for which a net increase in cartilage production would be beneficial can be treated. For example, any inflammatory or degenerative condition causing cartilage loss or damage can be treated, such as any type of arthritis, including osteoarthritis, rheumatoid arthritis, gout, and others. Other conditions include osteochondritis dissecans, avascular necrosis, chondromalacia, and others. *See, e.g.*, information provided by the Arthritis Foundation (www.arthritis.org) or the Wheeless' Textbook of Orthopaedics (<http://www.medmedia.com/med.htm>). In the case of avascular necrosis, for example, involving cartilage loss due to the death of the subchondral bone, the present methods would allow the regeneration of both cartilage and subchondral bone. Also treatable are cartilage defects resulting from an injury to the cartilage, *e.g.*, from major trauma, minor trauma, repeated minor trauma, surgery, *e.g.*, producing rotational force, shear force, *etc.*, and producing any type of lesion or damage, such as fibrillation, discoloration, softness, cracks, tears, craters, *etc.* Both full-thickness and partial-thickness defects are treatable using the present methods.

Kits

The invention also provides packs, dispenser devices, and kits for administering vectors of the invention to a mammal. For example, packs or dispenser devices that

contain one or more unit dosage forms are provided. Typically, instructions for administration of the nucleic acids will be provided with the packaging, along with a suitable indication on the label that the nucleic acid is suitable for treatment of an indicated condition. For example, the label may state that the active compound within the packaging is useful for treating osteoarthritis, or for preventing or treating other diseases or conditions that are associated with destruction of connective tissue.

EXAMPLE 1:

In vivo gene delivery into subchondral tissue was demonstrated in a rabbit model.

The rabbit knee was surgically exposed, and then a small hole was drilled into the femoral groove of the knee through the cartilage and into the subchondral bone. Type I and Type 2 collagen matrix was mixed with a solution containing a vector encoding firefly luciferase, and the mixture was implanted into the hole. The joint capsule, and then the skin, were sutured. At various time points after implantation, the rabbits were sacrificed and the collagen plugs removed along with surrounding tissue scraped out of the hole. The collagen/tissue plug was homogenized and analyzed for luciferase expression.

Data from experiments in which implants included an adenovirus vector are shown in Figure 1. When plasmid DNA was added, about 200 µg of DNA was used. With adenoviral formulations, about 1×10^7 pfu, \pm 5-fold was used. Using the adenovirus vector, luciferase was expressed and was able to persist at least three weeks.

Figure 2 shows the results of implantation of numerous non-viral formulations mixed with a Type I and Type 2 collagen matrix. The formulations shown in Figure 2 include: naked DNA (2 mg/ml plasmid DNA); MB192 -- 1:1 (DNA:cationic lipid) DOTIM/CHOLESTEROL Liposomes (2 mg/ml final DNA concentration); MB322 --1:1 (DNA:chitosan) Glycol chitosan - 1 mg/ml final DNA concentration; MB314 -- 3:1 (DNA:paromomycin) Paromomycin - 1 mg/ml final DNA concentration; MB306 -- 1:40:0.1:0.6 (DNA:Tributyrin:RGDS peptide:Brij 35 - 2 mg/ml DNA final concentration; and a control (Phosphate buffered saline (PBS)). As demonstrated in by the results presented in Figure 2, expression was achieved after 24 hours using naked DNA and cationic lipid formulations.

galactosidase (*lac Z* gene). Transduction was performed in 300 microliters of GBSS for 1 hour at 37°C at various multiplicities of infection (MOIs), as indicated. Following transduction, the supernatant was aspirated and replaced with 500 microliters of Dulbecco's modified Eagle's medium (DMEM) supplemented with 1% FBS and antibiotics.

Exogenous recombinant human IGF-1 (R&D Systems, Minneapolis, MN) was added to the media of the nontransduced control groups. Recombinant human IL-1-β (2 ng/ml) was also added to the appropriate groups at this time. Cells were then incubated for 48 hours before the addition of radionucleotides for the determination of matrix synthesis.

IGF-1 measurements.

The concentration of IGF-1 in cell supernatants was determined at 48 hours following transduction using a commercially available enzyme-linked immunosorbent assay (ELISA) kit and protocol (Diagnostics Laboratory Systems, Webster, TX).

Proteoglycan and collagen synthesis.

The synthesis of matrix molecules was measured by previously published methods (Taskiran, *et al.* (1994) *Biochem Biophys Res Commun* 200:142-8; Cao, *et al.* (1997) *Biochem J* 325:305-10.)

To measure proteoglycan synthesis, the media were aspirated following the initial 48-hour incubation period and replaced with 500 microliters of fresh DMEM with 1% FBS. Exogenous growth factors and IL-1 were also readministered to the appropriate wells. Each culture well received 20 microCi/ml of ³⁵S-labeled Na₂SO₄, and the cells were incubated for an additional 24 hours. The synthesis of proteoglycans was determined in the cell layer and the media by the incorporation of ³⁵SO₄²⁻ into glycosaminoglycans. Size-exclusion chromatography with PD10 columns (Pharmacia, Piscataway, NJ) was used to separate the glycosaminoglycans from unincorporated label.

Protein synthesis was determined by the incorporation of ³H-proline into collagen and noncollagenous proteins in the following manner. When the cells reached confluence, the medium was removed and replaced with serumless medium containing 50 micrograms/ml of ascorbic acid, 50 micrograms/ml of β-aminopropionitrile, and 20 microCi/ml of ³H-proline for 24 hours. After incubation, the conditioned medium and

cell layer were collected and combined, and the relative collagen synthesis was determined by ^3H -proline incorporation using a modified collagenase digestion method.

Briefly, after 24 hours of culture in the presence of the radioisotope, a carrier protein (2 mg/ml of pepsin-solubilized bovine type II collagen) was added, and the proteins were precipitated by addition of 10% trichloroacetic acid (TCA). The TCA-precipitated proteins were recovered by centrifugation at 14,000 revolutions per minute for 30 minutes at 4°C. The recovered protein pellets were washed repeatedly with 5% TCA to remove the unbound isotope. The washed pellets were resuspended in the collagenase buffer (5 millimolar CaCl_2 and 3 millimolar *N*-ethylmaleimide), the pH was adjusted to 7, and the suspensions were incubated at 37°C for 2 hours. TCA was then added, and the mixture was centrifuged for 30 minutes at 14,000 rpm. The supernatants were removed and were used as enzyme blanks. The pellets were resuspended in the collagenase buffer, the pH was adjusted to 7 by the addition of NaOH, and purified bacterial collagenase ABC form III (Advanced Biofactures, Lynbrook, NY) was added (20 units/ml) and incubated for 2 hours at 37°C.

The collagenase-digestible proteins were separated from nondigested proteins by the addition of 5% TCA followed by centrifugation at 14,000 rpm for 30 minutes. The pellets were redissolved in 0.2 M NaOH, and aliquots of the pellets and the radioactivities of the supernatants were measured to determine the level of collagen and noncollagenous protein synthesis. The data were corrected for cell number by counting cells in parallel wells that received the same factors and were plated at the same density as in the experimental wells.

Determination of collagen phenotype.

Collagen phenotyping was also performed on each sample, as described elsewhere (Cao, *et al.* (1997) *Biochem J* 325:305-10.) Briefly, following the 24-hour labeling period with ^3H -proline, all samples underwent pepsin (100 micrograms/ml) digestion for 4 hours. The pepsin-resistant chains were precipitated with 3 M NaCl followed by dialysis against NH_4HCO_3 for 5 days. Samples were then dried and separated by 7.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Gels were treated with EN 3 HANCE (DuPont NEN, Boston, MA) prior to autoradiography.

IGF-1 gene expression and matrix synthesis.

Resting cultures of rabbit articular chondrocytes secreted 3.9 ± 1.7 ng/ml (mean \pm SD) IGF-1 into their culture media. Infection with AdIGF-1 increased IGF-1 production

dramatically, in a dose-dependent manner as shown in Figure 3A. Experiments with the AdLacZ vector confirmed that at an MOI of 300, all chondrocytes had been transduced (results not shown); the number of viral genomes per cell under these conditions is not known. IGF-1 protein was added to certain cultures to confirm the accuracy of the ELISA.

Figure 3 shows the production of insulin-like growth factor 1 (IGF-1) by transduced chondrocytes and its effects on matrix synthesis. Figure 3A shows IGF-1 concentrations in conditioned media following transduction with the IGF-1 gene or the addition of IGF-1 protein. Cultures of chondrocytes were infected with AdIGF-1 at the indicated multiplicity of infection (MOI) or were supplemented with IGF-1 at the indicated dosages. After 48 hours, all conditioned media were assayed for IGF-1. Figure 3B shows effects of IGF-1 protein and gene on the synthesis of proteoglycans by chondrocytes. Incorporation of $^{35}\text{SO}_4^{2-}$ into proteoglycans present in the conditioned media and the cell layer was measured under the indicated conditions (columns are stacked). Figure 3C show the effects of IGF-1 protein and gene on the synthesis of collagen and noncollagenous proteins by chondrocytes. Incorporation of ^3H -proline into collagenase-sensitive and collagenase-resistant proteins was measured under the indicated conditions. Values are the mean and SD ($n = 6$). * = $P < 0.05$ versus control.

Chondrocytes were incubated with ^3H -proline under the conditions indicated. Pepsin-resistant proteins were then separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and subjected to autoradiography. Collagen typing confirmed that IGF-1, whether delivered as a protein or as a gene, maintained the phenotype of the chondrocytes. The predominant collagen produced by the cells under all conditions was type II collagen, with barely detectable amounts of $\alpha 2(\text{I})$, which would indicate the synthesis of type I collagen. A prominent band was seen at the position where the $\alpha 1(\text{V})$ and $\alpha 1(\text{XI})$ chains comigrate, indicating the presence of types V and XI collagens. Articular chondrocytes are known to produce both of these types of collagens.

Monolayer cultures of chondrocytes readily incorporated radiolabeled precursors into matrix macromolecules. In multiple experiments, the radioactivities of the various matrix molecules (mean \pm SD) were as follows: for proteoglycan in the cell layer $4,620 \pm 1,104$ disintegrations per minute, proteoglycan in the medium $7,465 \pm 692$ dpm, collagen $2,415 \pm 354$ dpm, and noncollagenous proteins $42,487 \pm 4,719$. Infection of chondrocytes with AdLacZ did not alter matrix synthesis by chondrocytes.

Both IGF-1 protein and transduction with IGF-1 cDNA enhanced proteoglycan synthesis ~2-3-fold as shown in Figure 3B. Due to biologic variability, statistical significance was only achieved at the highest concentration of IGF-1 protein and 300 MOI of virus. Proteoglycan synthesis declined at the highest MOI, despite the high levels of IGF-1 produced at this dose of virus. In all cases, increased proteoglycan deposition was noted in the cell layer, as well as in the conditioned media, suggesting that the newly synthesized proteoglycan produced in response to IGF-1 was incorporated into a stable, cell-associated matrix.

IGF-1 protein had a more modest effect on the synthesis of collagen and noncollagenous proteins by the chondrocytes, but the stimulatory effects of transducing the IGF-1 cDNA were very marked (Figure 3C). Even at an MOI of 100, where IGF-1 accumulated in the medium to ~80 ng/ml (Figure 3A), an approximately 3-fold increment was seen (Figure 3C). Addition of 100 ng/ml of protein, in contrast, achieved only about a 1.5-fold increment, despite the presence of ~160 ng/ml IGF-1 in the culture medium (Figure 3A).

Growth factor combinations.

Additive effects of growth factor (insulin-like growth factor 1 [IGF-1] and transforming growth factor β 1 [TGF β 1]) gene combinations on proteoglycan synthesis by chondrocytes were demonstrated as shown in Figure 4. Cultures of chondrocytes were infected with a combination of AdIGF-1 and AdTGF β 1 at the indicated multiplicity of infection (MOI). The incorporation of $^{35}\text{SO}_4^{2-}$ into proteoglycans present in the conditioned medium and cell layer was then measured (columns are stacked). Values are the mean and SD (n = 6). * = $P < 0.05$ versus control. A combination of AdIGF-1 and AdTGF β 1 was employed at 2 different MOIs. As Figure 4 demonstrates, growth factor gene combinations had an additive effect on proteoglycan synthesis.

Effects of IL-1

IL-1 inhibits proteoglycan synthesis by articular chondrocytes (Tyler, *et al.* (1985) *Biochem J* 227:869-78.) Both AdTGF β 1 and AdIGF-1 were able to reverse this effect as shown in Figure 5. Chondrocytes were transduced with AdTGF β 1 (Figure 5A) or AdIGF-1 (Figure 5B) at the indicated multiplicity of infection (MOI) and then incubated in the presence or absence of IL-1 for 48 hours. The incorporation of $^{35}\text{SO}_4^{2-}$ into proteoglycans present in the conditioned media or the cell sheet was then measured

(columns are stacked). In the case of AdTGF β 1, incorporation of $^{35}\text{SO}_4^{2-}$ into only the total culture (media + cells) was measured. Values are the mean and SD ($n = 6$). $*=P < 0.05$ versus control cells incubated with IL-1.

The expression of all transgenes was high following adenoviral transduction. Proteoglycan synthesis was stimulated ~2–3-fold by the IGF-1 gene. Synthesis of collagen and noncollagenous proteins, in contrast, was most strongly stimulated by the IGF-1 gene. Collagen typing confirmed the synthesis of type II collagen. IL-1 suppressed proteoglycan synthesis by 50–60%. IGF-1 and TGF β genes restored proteoglycan synthesis to control levels in the presence of IL-1.

While the foregoing invention has been described in some detail for purposes of clarity and understanding, it will be clear to one skilled in the art from a reading of this disclosure that various changes in form and detail can be made without departing from the true scope of the invention. For example, all the techniques and apparatus described above may be used in various combinations. All publications and patent documents cited in this application are incorporated by reference in their entirety for all purposes to the same extent as if each individual publication or patent document were so individually denoted.

WHAT IS CLAIMED IS:

- 1 1. A method of providing a heterologous protein in a subchondral cell
2 in a joint of a mammal, comprising:
3 (i) creating or locating a perforation in a subchondral bone of said joint;
4 (ii) introducing a polynucleotide encoding said heterologous protein into said
5 perforation, whereby a subchondral cell localized to the perforation internalizes the
6 polynucleotide and expresses the heterologous protein.
- 1 2. The method of claim 1, wherein said perforation is produced by
2 drilling.
- 1 3. The method of claim 1, wherein said perforation comprises a
2 microfracture.
- 1 4. The method of claim 1, wherein said perforation is produced by
2 abrasion.
- 1 5. The method of claim 1, wherein said polynucleotide is present
2 within an artificial matrix.
- 1 6. The method of claim 5, wherein said matrix comprises collagen.
- 1 7. The method of claim 1, wherein said heterologous protein is a
2 chondrogenic polypeptide.
- 1 8. The method of claim 7, wherein said chondrogenic polypeptide is a
2 growth factor.
- 1 9. The method of claim 8 wherein said growth factor is TGF- β .
- 1 10. The method of claim 8 wherein said growth factor is IGF-1.
- 1 11. The method of claim 7, wherein said chondrogenic polypeptide is a
2 cartilage-derived morphogenetic factor.
- 1 12. The method of claim 1, wherein said heterologous protein is a
2 marker gene.

- 1 13. The method of claim 12, wherein said marker gene is β -
2 galactosidase.
- 1 14. The method of claim 1, wherein said polynucleotide is delivered
2 using an adenoviral vector.
- 1 15. The method of claim 1, wherein said polynucleotide is delivered
2 using formulated plasmid DNA.
- 1 16. The method of claim 1, wherein said polynucleotide is delivered
2 using naked plasmid DNA.
- 1 17. A method of providing a plurality of growth factors in a
2 subchondral cell in a joint of a mammal, comprising:
3 (i) creating or locating a perforation in a subchondral bone of said joint;
4 (ii) introducing one or more polynucleotides encoding said plurality of growth
5 factors into said perforation, whereby a subchondral cell localized to the perforation
6 internalizes the polynucleotide or polynucleotides and expresses the growth factors.
- 1 18. The method of claim 17, wherein said plurality of growth factors
2 comprises IGF-1 and TGF- β .
- 1 19. A medicament for use in localizing a polynucleotide to a joint of an
2 animal, said medicament comprising a mixture of collagen and said polynucleotide.
- 1 20. The medicament of claim 19, wherein said medicament is used to
2 localize said polynucleotide to a subchondral perforation within said joint.
- 1 21. The medicament of claim 19, wherein said polynucleotide is
2 complexed with a cationic lipid.
- 1 22. The medicament of claim 19, wherein said polynucleotide encodes
2 a chondrocyte or chondroprogenitor growth factor.
- 1 23. The medicament of claim 19, wherein said polynucleotide encodes
2 a cytokine.

1 24 The medicament of claim 19, wherein said polynucleotide encodes
2 a cytokine inhibitor.

1 25. A medicament for use in expressing a polynucleotide in a joint of
2 an animal, said medicament comprising:

(a) a polynucleotide capable of functionally expressing a desired protein in subchondral cells within said joint; and

5 (b) a pharmaceutically acceptable diluent,

6 wherein the medicament is delivered to a perforation in a bone within said joint
7 and results in expression of said polynucleotide in said subchondral cells.

1 26. The medicament of claim 25, further comprising a collagen matrix.

1 27. The medicament of claim 25, wherein said polynucleotide is
2 complexed with a cationic lipid.

1 28. The medicament of claim 25, wherein said polynucleotide encodes
2 a chondrocyte or chondroprogenitor growth factor.

1 29. The medicament of claim 25, wherein said polynucleotide encodes
2 a cytokine.

1 30. The medicament of claim 25, wherein said polynucleotide encodes
2 a cytokine inhibitor.

1 31. The medicament of claim 25, wherein said medicament comprises
2 a plurality of polynucleotides encoding a plurality of growth factors, wherein said
3 plurality of growth factors acts synergistically to stimulate matrix synthesis.

1 32. The use of collagen and a polynucleotide encoding a protein of
2 interest to obtain expression of said protein in a subchondral cell of an animal joint,
3 wherein said collagen and said polynucleotide are introduced into a subchondral
4 perforation within said joint.

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- (54) Title:** METHODS FOR *IN VIVO* GENE DELIVERY TO SITES OF CARTILAGE DAMAGE

- (57) Abstract:** The present invention provides methods for *in vivo* gene therapy methods for the treatment of joint diseases, such as osteoarthritis. The methods involve the exposure of subchondral cells in a joint and the subsequent transfection of these cells *in vivo*.

Figure 1

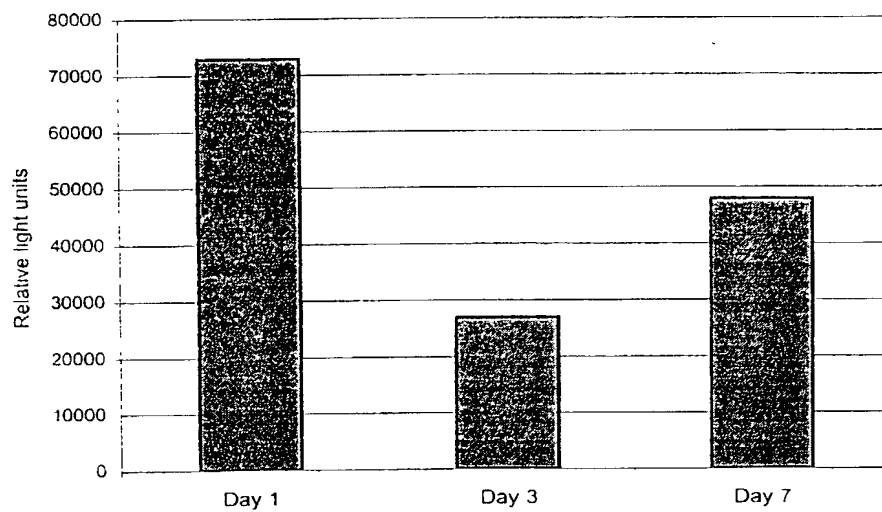
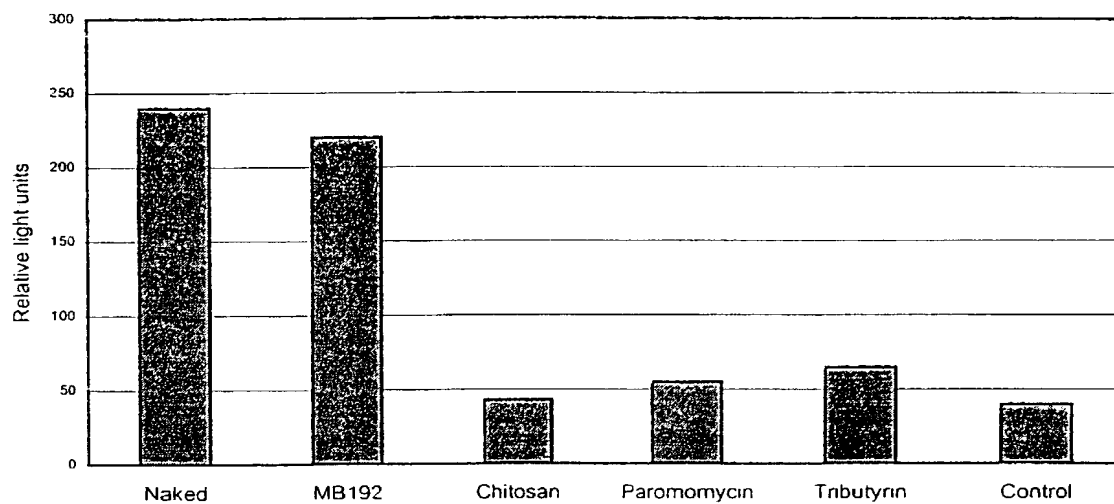
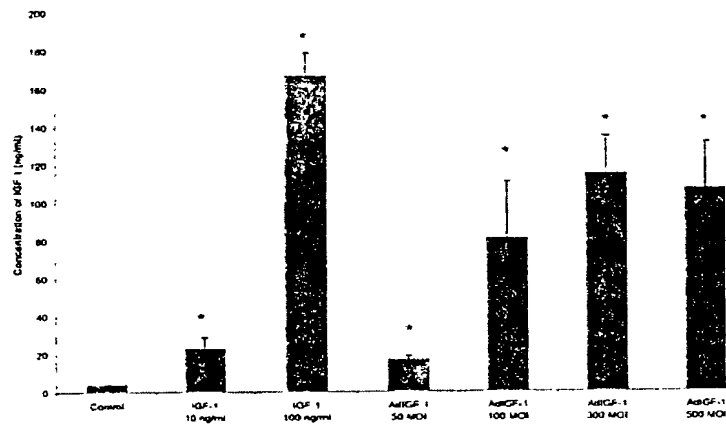


Figure 2



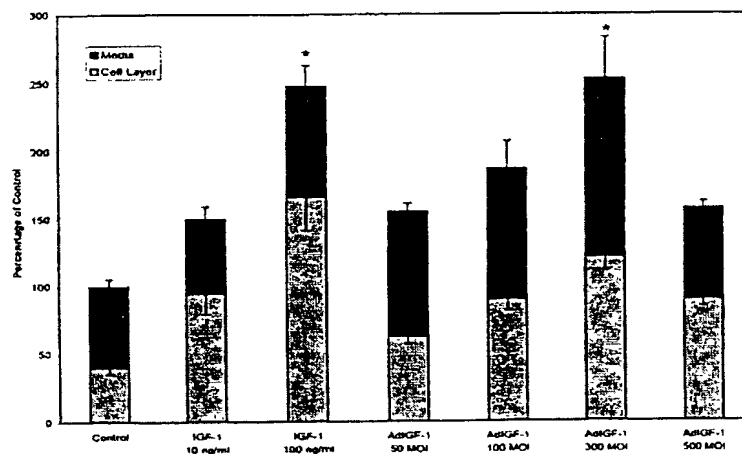
3/5

Figure 3A



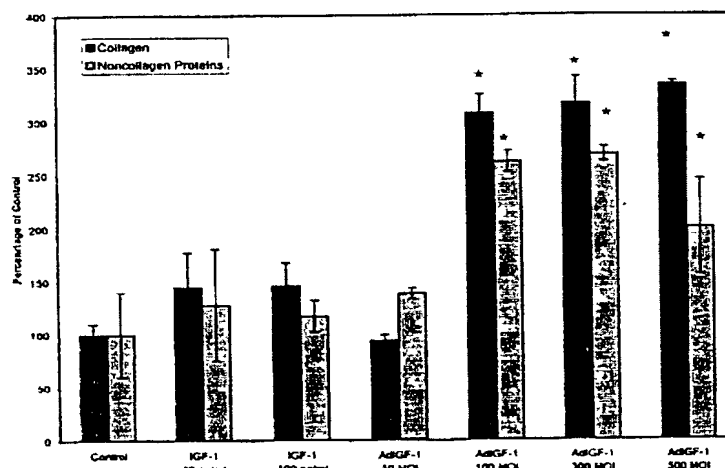
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Figure 3B



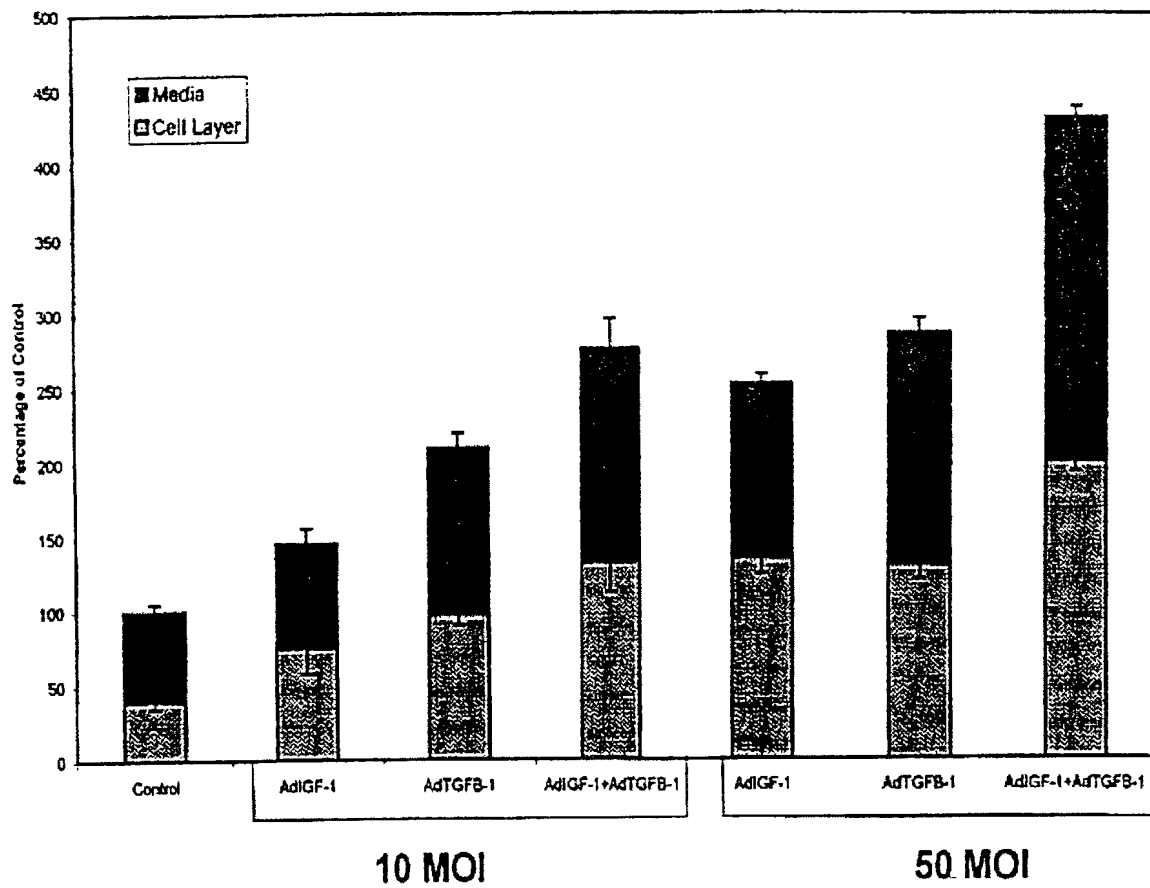
B

Figure 3C



C

Figure 4



5/5

Figure 5A

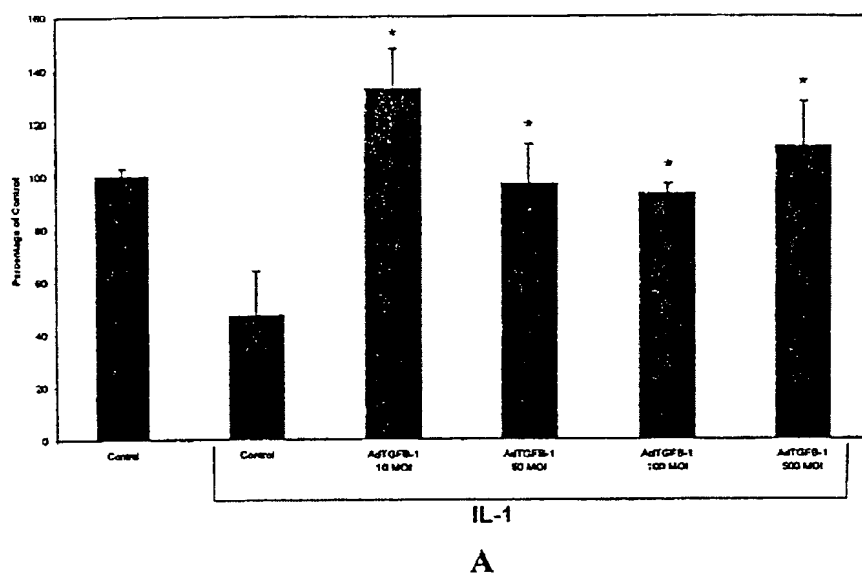
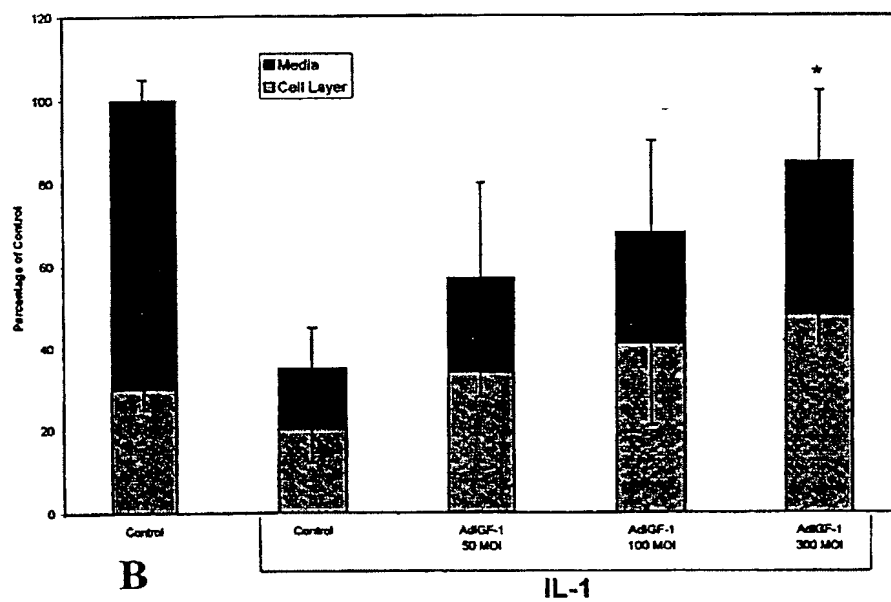


Figure 5B



Attorney Docket No.: 018484-002320US

Client Ref. No.:

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DECLARATION (37 CFR 1.63) FOR UTILITY OR DESIGN APPLICATION USING AN APPLICATION DATA SHEET (37 CFR 1.76)

As the below named inventor(s), I/we declare that:

This declaration is directed to:

- ☐ The attached application, or
- ☒ Application No. 10/069323 filed on February 20, 2002
- ☐ as amended on _____ (if applicable);

I/we believe that I/we am/are the original and first inventor(s) of the subject matter which is claimed and for which a patent is sought;

I/we have reviewed and understand the contents of the above-identified application, including the claims, as amended by any amendment specifically referred to above;

I/we acknowledge the duty to disclose to the United States Patent and Trademark Office all information known to me/us to be material to patentability as defined in 37 CFR 1.56, including material information which became available between the filing date of the prior application and the National or PCT International filing date of the continuation-in-part application, if applicable; and

All statements made herein of my/our own knowledge are true, all statements made herein on information and belief are believed to be true, and further that these statements were made with the knowledge that willful false statements and the like are punishable by fine or imprisonment, or both, under 18 U.S.C. 1001, and may jeopardize the validity of the application or any patent issuing thereon.

FULL NAME OF INVENTOR(S)Inventor 1 1-00 Steven C. Ghivizzani Date: 4/4/02Signature: [Signature] Citizen of: United StatesInventor 2 2-00 Christopher H. Evans Date: _____Signature: _____ Citizen of: United KingdomInventor 3 3-00 Paul D. Robbins Date: _____Signature: _____ Citizen of: United States

Inventor 4 _____ Date: _____

Signature: _____ Citizen of: _____

☐ Additional inventors are being named on form(s) attached hereto.

Burden Hour Statement: This collection of information is required by 35 U.S.C. 115 and 37 CFR 1.63. The information is used by the public to file (and the PTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This form is estimated to take 1 minute to complete. This time will vary depending upon the needs of the individual case. Any comments on the amount of time you are required to complete this form should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, Washington, DC 20231. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Assistant Commissioner for Patents, Washington, DC 20231.

SF 1329080 v1

Attorney Docket No.: 018484-002320US
Client Ref. No.:

PTO/SB/01A (10-00)

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FULL NAME OF INVENTOR(S)

Inventor 1 Steven C. Ghivizzani Date: _____

Signature: _____ Citizen of: United States

Inventor 2 Christopher H. Evans Date: 5.8.02

Signature: CH Evans Citizen of: United Kingdom

Inventor 3 Paul D. Robbins Date: _____

Signature: _____ Citizen of: United States

Inventor 4 _____ Date: _____

Signature: _____ Citizen of: _____

☐ Additional inventors are being named on form(s) attached hereto.

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FULL NAME OF INVENTOR(S)

Inventor 1 Steven C. Ghivizzani Date: _____Signature: _____ Citizen of: United StatesInventor 2 Christopher H. Evans Date: _____Signature: _____ Citizen of: United KingdomInventor 3 Paul D. Robbins Date: 4/8/02Signature: Paul D. Robbins Citizen of: United States

Inventor 4 _____ Date: _____

Signature: _____ Citizen of: _____

☐ Additional inventors are being named on form(s) attached hereto.

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